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ISOLATION OF A CRYSTALLINE TOXIC FACTOR FROM AGENIZED WHEAT FLOUR

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IN 1946 it was established by Mellanby[1] that dogs fed a diet rich in commercially 'improved' (nitrogen trichloride-treated) flour were subject to epileptiform fits; similar fits were never seen in control animals fed on untreated flour. Mellanby concluded that the treatment of flour with nitrogen trichloride gave rise to a toxic factor which was responsible for the condition in dogs well known in Great Britain as 'canine hysteria' and in the United States as 'running fits'.

A large proportion of the flour milled in Britain and used for bread-making is 'improved' by treatment with nitrogen trichloride and is known commercially as 'agenized' flour. The observation that this flour was toxic to dogs was followed by a demonstration that it was also toxic to ferrets[2], to cats[3], and to rabbits[4]. It thus became a matter of some importance to discover the nature of the toxic factor and to assess the possible danger which might arise from the use of agenized flour.

Method of Test

Throughout the present investigation the criterion of toxicity adopted has been the production of a typical epileptiform fit in the ferret. The ferret is slightly less sensitive than the dog on a body-weight basis; but the small size of the animal enabled us to obtain a toxic reaction with a much smaller dose of agenized flour than would be necessary for a dog.

The demonstration by Mellanby[2] that toxicity was confined to the gluten fraction of the flour suggested that the toxic factor might be a modified amino-acid or peptide. The final method of isolation which is outlined below was based on this assumption.

Isolation of Crystalline Toxic Factor

Flour was agenized by the standard procedure but with ten times the amount of nitrogen trichloride used in commercial practice (11·1 gm. NCl_3 to 17·7 kgm. flour). This flour was toxic to a ferret in

a dose of 100 gm. (1·9 gm. nitrogen) fed over three days. Gluten was separated from the flour and digested first with pepsin and then with trypsin. The digest was dialysed and the dialysate hydrolysed for four hours with strong hydrochloric acid. The acid was removed and the hydrolysate electrodialysed. The neutral fraction from the electrodialysis was treated with sufficient charcoal to remove the aromatic amino-acids[5], and the residual neutral amino-acid mixture was then fractionated on a 'Zeokarb 215' column by the method of Partridge[6]. A fraction was obtained from the 'Zeokarb' column which produced a typical fit in a ferret when fed for three days at a level of 17 mgm. nitrogen per day. This active material contained at least fifteen components and was further fractionated by chromatography on a paper column ('Solka Floc') using a mixture of butanol, acetic acid and water as solvent. The toxic factor crystallized when the appropriate fraction of the effluent was concentrated.

Toxicity

The crystalline toxic factor (see accompanying illustration) produced severe epileptiform fits in a ferret when fed for five days at a level of 100 μgm. nitrogen per day; this represents a total dose of crystalline material of approximately 3 mgm. The pure toxic factor is thus 33,000 times as toxic as the original flour.

When fed as a single dose the crystalline material is slightly more toxic than when the dose is spread over several days. A single dose of 2 mgm. is sufficient to produce typical epileptiform fits in a ferret, and a slightly greater dose kills the animal. Particular emphasis must be laid, however, on the cumulative nature of the poisonous effect. We have demonstrated in the ferret that a fatal toxic reaction can be produced by feeding the concentrated toxic factor in small doses over a period of five to ten days, and that the daily intake in such cases is only a fraction of the dose required to produce an immediate toxic response. It must be added, however, that this toxic action is not a simple problem and is probably influenced by other elements in the diet.

Nature of the Toxic Factor

As the amount of toxic factor in flour is small (1 gm. per 33,000 gm. flour) and the isolation procedure gives an overall yield of only 10 per cent, the greatest difficulty has been experienced in obtaining sufficient material for combined biological and chemical characterization. All the results reported below have been obtained on about 10 mgm. of crystalline toxic factor.

Crystalline toxic factor from agenized flour (× 180, phase contrast)

We were initially of the opinion that the toxic factor was a peptide, since a sample hydrolysed for twenty-four hours with 6*N* HCl at 110° and examined by partition chromatography[7] in two dimensions on paper was found to give six ninhydrin-positive spots. One of these spots was, however, identified as the toxic factor itself, and even forty-eight hours hydrolysis did not destroy it entirely. As a peptide could scarcely be expected to resist such vigorous hydrolytic conditions some doubt arose in our minds as to the peptide nature of our material.

A specimen of toxic factor hydrolysed for 24 hr. with 6*N* hydrochloric acid was studied in detail and the following degradation products were provisionally identified by paper chromatography : homocysteic acid, α-aminobutyric acid, methionine sulphoxide, methionine sulphone and homoserine. Since all these compounds can be regarded as possible degradation products of a molecule containing the methionine skeleton, we concluded that our toxic factor was probably derived from methionine. A small sample of the crystalline material was desulphurized by treatment with Raney nickel[8], and the product identified by paper chromatography as α-aminobutyric acid ; methionine gave α-aminobutyric acid under similar conditions.

It might be thought from the above evidence that the toxic factor was produced from methionine by a simple reaction with nitrogen trichloride. However, this would not appear to be so, for Silver[9] has shown

that when methionine is treated with nitrogen trichloride the toxic factor is not produced. We have recently treated the following peptides (kindly supplied by Dr. Fruton) with nitrogen trichloride: methionyl - glycine, carbobenzoxymethionyl - methionine amide and carbobenzoxymethionyl - methionine, and were unable to detect the presence of the toxic factor by partition chromatography after either a 4- or 16-hr. hydrolysis with strong hydrochloric acid at 110° C.

Comparison of Toxic Factors from Wheat Flour and Zein

Dr. Reiner, of Wallace Tiernan and Co., has recently kindly supplied us with a sample of a crystalline toxic factor which had been isolated from zein treated with nitrogen trichloride. Our crystalline material was indistinguishable from that of Dr. Reiner when examined by two-dimension partition chromatography on paper. The melting points of the two materials were within the same range, and both materials, fed to ferrets, produced the same toxic reaction.

While this work was in progress three communications[10] appeared from the laboratory of the British Flour-Millers Research Association, St. Albans, on a toxic factor present in nitrogen trichloride-treated zein. Although we have not been able to carry out a direct comparison between our own material and that isolated by this group, we find that the published details on the properties of the crystalline material isolated at St. Albans are in close agreement with our own. It seems highly probable, therefore, that the material isolated by us from agenized flour is identical with the material isolated by Dr. Reiner and independently by the St. Albans group from nitrogen trichloride-treated zein. We are indebted to Mr. J. Smiles for the photograph of the crystalline toxic factor and to Dr. C. Dent for samples of homoserine and α-aminobutyric acid.

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